

# Evaluations of Cellulose Accessibilities of Lignocelluloses by Solute Exclusion and Protein Adsorption Techniques

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**ABSTRACT:** Cellulose accessibilities of a set of hornified lignocellulosic substrates derived by drying the never dried pretreated sample and a set of differently pretreated lodgepole pine substrates, were evaluated using solute exclusion and protein adsorption methods. Direct measurements of cellulase adsorption onto cellulose surface of the set of pretreated substrates were also carried out using an in situ UV–Vis spectrophotometric technique. The cellulose accessibilities measured by the solute exclusion and a cellulose-binding module (CBM)-containing green fluorescent protein (TGC) adsorption methods correlate well for both sets of samples. The substrate enzymatic digestibilities (SEDs) of the hornified substrates are proportional to the measured cellulose accessibilities. Approximately over 90% of the SED was contributed by the accessible pore surfaces of the hornified substrates, suggesting that the substrate external surface plays a minor role contributing to cellulose accessibility and SED. The cellulose accessibilities of the pretreated substrates correlated well with the amounts of cellulase adsorbed. The SEDs of these substrates directly correlated with the amounts of adsorbed cellulase.

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**KEYWORDS:** cellulose accessibility to cellulase; enzymatic hydrolysis/saccharification; protein/cellulose adsorption; fiber hornification; pretreatment

## Introduction

Lignocellulose is the most abundant polymeric carbohydrate in the world that could be used for sustainable biofuel production (Mielenz, 2001; Sun and Cheng, 2002). The efficient bioconversion of lignocellulosic feedstock to sugars and cellulosic biofuel involves three key steps: feedstock pretreatment, enzymatic saccharification, and catalytic conversion or fermentation. Enzymatic saccharification has been identified as one of the most costly steps in cellulosic ethanol production (Lynd et al., 2008). The intimate contact between the cellulose and cellulase is the prerequisite step for enzymatic cellulose hydrolysis to take place, therefore the cellulose accessibility to cellulase (CAC) is critical. Lignocellulosic biomass is made of approximately 60% of noncellulosic components such as hemicelluloses and lignin that form a strong composite with cellulose to prevent cellulose accessible to enzymes and microbes for deconstruction. Therefore, increasing the CAC is particularly critical to improve enzymatic hydrolysis (Jeoh et al., 2007; Rollin et al., 2011). The determination of cellulose accessibility can help to understand the effectiveness of a particular pretreatment in removing lignocellulose recalcitrance to improve substrate enzymatic digestibility (SED).

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Cellulose accessibility of a lignocellulosic substrate has been evaluated by several methods. These methods can be categorized into two general approaches. The first approach directly measures the physical accessible volume (porosity) or surface of a substrate using one or a set of probing molecules, such as water molecule in the water retention value (WRV) method (Luo and Zhu, 2011) and the differential scanning calorimetric and NMR porosimetry (Li et al., 1997; Maloney et al., 1998; Felby et al., 2008), or a set of dextran molecules in the classical solute exclusion technique (Stone and Scallan, 1967; Hui et al., 2009). A summary of the methods of the first approach can be found in a recent book chapter (Beecher et al., 2009). The second approach measures the adsorption of a given molecule to a lignocellulosic substrate, which includes the BET method that measures the adsorption of nitrogen by the pore surfaces (Brunauer et al., 1938; Chen et al., 2010), Simons' staining method that measures the adsorption of dyes by the lignocellulosic substrate (Esteghlalian et al., 2001; Chandra et al., 2008), and protein or cellulase adsorption method that directly measures the amount of protein or cellulase adsorbed onto a lignocellulosic substrate (Hong et al., 2007; Zhu et al., 2009b; Luo et al., 2011).

The WRV and BET methods both suffer from the fact that molecular size of water and nitrogen is much smaller than cellulase enzymes, which can result in overpredicting the cellulose accessibility to cellulase. Furthermore, the BET method requires the substrate to be dried, as a result, the measured pore surface differs significantly from that under wet state during enzymatic hydrolysis in a suspension. The Simons' staining method slightly alleviated the problem using two dye molecules; however, it cannot provide good measurement of pore size distribution. Furthermore, the cellulose accessibility based on dye adsorption is different from those determined using enzymes because the molecular sizes of the dyes are often significantly different from those of enzymes. Furthermore, dyes do not have a cellulose-binding module (CBM) as cellulase has. The solute exclusion method can provide a good measurement of pore size distribution. It can be used to determine CAC when the effective enzyme molecular size is known. But solute exclusion cannot determine accessibility of external surfaces (Hong et al., 2007). All the studies discussed above except for the work by Hong et al. (2007) did not involve a CBM to interact with cellulose and measured the total exposed surface/volume to the probe molecules. These surfaces can be noncellulosic, for example, hemicelluloses and lignin. These noncellulosic components also have strong affinity to cellulase enzymes to produce nonproductive adsorption (Sewalt et al., 1997; Mansfield et al., 1999; Liu et al., 2010; Qing et al., 2010). Bovine serum albumin (BSA), surfactants, and metal salts were found effective to reduce or eliminate nonproductive adsorption of cellulase onto lignocelluloses (Eriksson et al., 2002; Yang and Wyman, 2006; Zheng et al., 2008; Liu and Zhu, 2010; Liu et al., 2010). It was demonstrated that the application of BSA before conducting protein adsorption measurements, can

effectively block cellulase adsorption onto lignin (Zhu et al., 2009b). The CAC thus determined represents only the accessible cellulose surface to cellulase, rather than the total accessible lignocellulosic surface. Furthermore, Zhu et al. (2009b) used a family 3 CBM-containing fusion protein (thioredoxin-green fluorescent protein-CBM3, TGC) as the probing molecule (Hong et al., 2007), which may have similar molecular size to that of cellulase enzymes.

The objectives of the present study are: (1) to evaluate cellulose accessibility measurements using the solute exclusion and protein adsorption techniques, including direct measurements of the adsorption of cellulase onto cellulosic surfaces of lignocelluloses by UV-Vis spectrophotometry (Liu et al., 2011); and (2) to conduct preliminary investigation of the relationship between cellulose accessibility and SED using a set of simple substrates with identical chemical composition but with significant variations in CAC, as well as a set of differently pretreated softwood substrates with significant differences in enzymatic digestibility.

## Materials and Methods

### Enzyme and Chemicals

Commercial cellulase enzymes, Fibercare<sup>®</sup>, an exoglucanase deficient endoglucanase, and Celluclast 1.5 L were generously provided by Novozymes of America, Franklinton, NC, and used as received. Novozyme 188  $\beta$ -glucosidase was purchased from Sigma-Aldrich (St. Louis, MO). BSA (Food grade, SeraCare, Milford, MA) was used as standard to calibrate the protein content of cellulase enzymes. The protein concentration of the Fibercare<sup>®</sup> solution was 6.476 mg/mL. All other chemicals used were of analytical grade unless otherwise stated.

### Substrates

All substrates were produced at the USDA Forest Service, Forest Products Laboratory, Madison, WI. Lodgepole pine (*Dendroctonus ponderosae*) was used to produce two sets of lignocellulosic substrates. The first set of five substrates was produced by air and heat drying handsheets (40 g/m<sup>2</sup>) made of a never dried (ND) pretreated lodgepole pine sample (PLPS) as described previously (Luo and Zhu, 2011). The air-drying was conducted in a humidity-controlled environment at 25°C with relative humidity (RH) of 50% for 24 h (AD). The heat drying was conducted by laying handsheet on a heated plat dryer (Model A-310, Adirondack Machine Corp., Glens Falls, NY) at 150°C for different periods of time of 1 (HD-1), 20 (HD-20), 30 (HD-30) min. The ND substrates were the same ND substrates (PLPS) described in our previous study (Luo and Zhu, 2011) and produced by disk milling of pretreated lodgepole pine wood chips by sulfite pretreatment to overcome recalcitrance of

lignocelluloses—SPORL (Zhu et al., 2009a). This set of substrates is chemically identical but varies significantly in pore surfaces due to drying-induced fiber hornification as will be discussed later. The second set of four substrates was obtained by disk milling untreated and pretreated lodgepole pine wood chips using either dilute sulfuric acid (DA), SPORL at high pH (4.2), or SPORL at low pH (1.9) pretreatments. These are the same substrates as those described in our previous study (Zhu et al., 2010). All three pretreatments were conducted at 180°C using liquid to wood ratio of 3:1 for 30 min. The sulfuric acid and sodium bisulfite charges for all pretreatments are listed in Table I. The chemical compositions of the resultant substrates were measured by the Analytical and Microscopy Laboratory of USDA Forest Products Laboratory (Table I).

Both sets of substrates were shipped to the University of New Brunswick, Canada, to determine substrate pore volume distributions using the solute exclusion technique. Identical sets of substrates were also shipped to the Virginia Polytechnic Institute for CAC determinations using the TGC adsorption method. The set of hornified substrates were rewetted by disintegrating several handsheets in 1 L deionized water after 10,000 revolutions at 312 rpm using a disintegrator (Model 73-06-01, TMI, Ronkonkoma, NY) as described previously (Luo and Zhu, 2011). Handsheets were made using the rewetted hornified substrates. The freshly made handsheets were immediately sealed in plastic zip lock bags to prevent the loss of moisture and forming hydrogen bonding to cause fiber hornification during shipping. The set of pretreated substrates (without making handsheets) was first frozen at -16°C and then shipped.

### Measurements of Substrate Water Retention Value (WRV)

The WRV of a substrate can represent the total water in the pores of a substrate or the total pore volume. The WRV of all substrates were measured following Scandinavian test method SCAN-C 62:00 (SCAN, 2000). As described in our previous study (Luo and Zhu, 2011), a hornified substrate was first rewetted in a disintegrator into suspension. The resultant suspension was carefully filtered using a nylon membrane (Sartolon Polymid, pore size 0.45 μm, Sartorius Stedim Biotech GmbH, Goettingen, Germany). The filter cake was added to deionized water to

make a suspension of about 10% solids consistency. After soaking for approximately 2 h, the suspension was wrapped by a nylon screen with mesh opening of 100 μm (Cole-Parmer, Vernon Hills, IL) and placed into a centrifuge tube with support to make space for water accumulation during centrifuge. The wrapped suspension was centrifuged at 3,000g for 15 min in a laboratory centrifuge (Thermo Fisher Scientific, Sorvall Legend 40/40R, Waltham, MA). WRV of the substrate is simply the amount of water retained after centrifuging as a percentage of the substrate dry weight. The averages of replicate measurements were used.

### Solute Exclusion for Pore Size Distribution Measurements

The solute molecules used were a series of dextran fractions obtained from Pharmacia AB, (Stockholm Sweden), together with glucose obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO). The relationship between molecular weights and molecular diameters used in this investigation is listed in Table II. The same experimental procedures and data analysis technique to those by Stone and Scallan (1967) (Hui et al., 2009) were followed. The specific volume of the pores inaccessible to a given probe molecule,  $V_{\text{inac},m}$  was directly determined from the measured concentration of the probe molecule (solute). Then the specific volume of the pores accessible to the given probe molecule,  $V_{\text{ac},m}$  was simply the specific volume of all pores,  $V_{\text{all}}$ , subtract the specific volume of inaccessible pores, that is,  $V_{\text{ac},m} = V_{\text{all}} - V_{\text{inac},m}$ . The specific volume of all pores is assumed to equal to the specific volume of the pores inaccessible to the largest probe molecule used. Therefore,

$$V_{\text{ac},m} = V_{\text{inac},560} - V_{\text{inac},m} \quad (1)$$

The cellulose accessibility to a particular probe molecule is equal to the specific accessible pore surface using the solute exclusion technique. By assuming all pores are cylindrical shape, the cellulose accessibility to a given probe molecule of size  $m$  is simply

$$\text{CAP}_m = \frac{4V_{\text{ac},m}}{m} = \frac{4(V_{\text{inac},560} - V_{\text{inac},m})}{m} \quad (2)$$

**Table I.** Pretreatment chemical dosages on oven dry wood and the chemical compositions of the resultant five lignocellulosic substrates used in this study.

Samples	Acid charge	Sodium bisulfite charge	Solids removal (%)	K Lignin	Arabinan	Galactan	Rhamnan	Glucan	Xylan	Mannan
Never dried SPORL substrate	0	8	35.0	32.00	ND	0.05	0.10	53.09	2.94	1.75
Pretreated lodgepole pine										
Untreated			0	27.01	1.56	2.23	0.7	42.55	6.93	10.99
Dilute acid	2.2	0	28.6	36.82	ND	ND	ND	49.84	0.16	0.08
SPORL high pH	0	8	31.1	32.84	ND	0.03	ND	59.86	2.35	1.12
SPORL low pH	2.2	8	33.3	38.13	0.01	0.07	ND	57.26	0.48	0.37

ND, not detectable.

**Table II.** List of molecules and their molecular weights and diameter used in the solute exclusion method.

Probe molecules	Average molecular weights	Diameter (Å)
D-Glucose	160	8
Dextran D9260	9300	51
Dextran T40	40,000	90
Dextran D1390	73,000	120
Dextran T500	500,000	270
Dextran T2000	2,000,000	560

The cellulose accessibility to cellulase, CAC, can be determined using a probe molecule whose size is equal to the molecular size of the cellulase when it is known, for example,  $m = 51 \text{ \AA}$  (Cowling and Kirk, 1976).

### TGC Adsorption for CAC Measurements

Total substrate accessibility to cellulase (TSAC,  $\text{m}^2/\text{g}$  substrate) was also measured based on the maximum adsorption capacity of cellulose for a nonhydrolytic fusion protein named TGC, containing a green fluorescent protein and CBM (Zhu et al., 2009b). The maximum binding capacities based on the adsorption of TGC for different cellulosic materials can vary greatly more than one order of magnitude (Liao et al., 2011). The recombinant TGC protein made in house was expressed in *Escherichia coli* BL21 (pNT02), and purified via an affinity-adsorption-based approach as described elsewhere (Hong et al., 2008). The TGC protein in 50 mM sodium citrate buffer (pH 6.0) solution was re-concentrated using a 10,000 Da molecular weight cut-off centrifugal ultrafiltration column (Millipore, Billerica, MA). The nonadsorbed TGC protein was measured using a BioTek multi-detection microplate reader, as described elsewhere (Hong et al., 2007). Therefore, the bound TGC amount equaled to that of initially added TGC minus nonbound TGC. Cellulose accessibility to cellulase (CAC,  $\text{m}^2/\text{g}$  substrate) can be measured based on the maximum TGC adsorption capacity after a blocking adsorption with a large amount of BSA (e.g., 5 g/L).

### Cellulase Adsorption for Cellulose Accessibility Measurements

Cellulose accessibility to cellulase was also evaluated by measuring the adsorption of a commercial endoglucanase, Fibercare<sup>®</sup>, using a UV-Vis spectrophotometric method (Liu et al., 2011). The technique corrected for cellulase adsorption by the leached lignin using a dual-wavelength method. The adsorption by noncellulosic components on the solid substrate was blocked by first applying BSA in the substrate suspension at 5 g/L (Zhu et al., 2009b). Approximately 30 mL of substrate suspension containing 1 g/L glucan and 5 g/L BSA in 50 mM acetate buffer at pH 4.8 were well mixed using a magnetic stir at a speed of 200 rpm at 25°C. After approximately 1 h mixing,

0.36 mL Fibercare<sup>®</sup>, which is equivalent to an endoglucanase concentration of 77.7 mg/L, was added to the substrate suspension. The protein concentration in the solution was continuously monitored by a UV-vis spectrophotometer (model 8453 Agilent, Palo Alto, CA) with a glass cuvette of 1 mm optical path length using absorption at 280 nm. The detailed description of the technique and measurement procedures can be found elsewhere (Liu et al., 2011). Cellulase adsorption experiments were at least duplicates. The average data were reported and the standard deviations were used as error bars.

### Enzymatic Hydrolysis

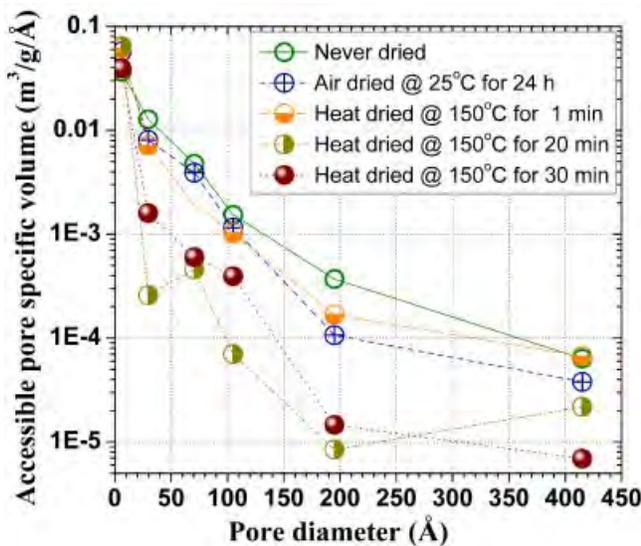
Before conducting enzymatic hydrolysis experiments, the set of hornified substrates was first rewetted in a disintegrator as described previously (Luo and Zhu, 2011). This is to eliminate the effect of fiber-flock size on enzymatic hydrolysis. Hydrolysis was conducted at 2% substrate solids (w/v) in 50 mM acetate buffer, pH 4.8, with 50 ppm tetracycline as antibiotic. Cellulase loading was 10 FPU/g glucan with  $\beta$ -glucosidase loading of 15 CBU/g glucan for all hydrolysis experiments. The solid substrate suspension was incubated on a shaker (Thermo Fisher Scientific, Model 4450) at 50°C and 200 rpm. Hydrolysate was sampled periodically, and glucose concentration was determined in replicate using a commercial glucose analyzer (YSI 2700S, YSI Inc., Yellow Springs, OH).

## Results and Discussion

### Cellulose Accessibilities and Enzymatic Hydrolyses of Hornified Substrates

It is well known that drying of delignified fibers can cause fiber hornification that refers to the irreversible loss of water binding ability upon drying of cellulose (Jayme, 1944). It is a consequence of the irreversible change of cell wall structure due to the collapse of pores (Stone and Scallan, 1965; Laivins and Scallan, 1993; Fernandes Diniz et al., 2004). Therefore, the set of hornified substrates produced from the same ND substrates will have substantial differences in accessible cellulose surfaces to cellulase through the pores. These substrates are suitable for evaluating different techniques for cellulose accessibility determination as they are chemically identical.

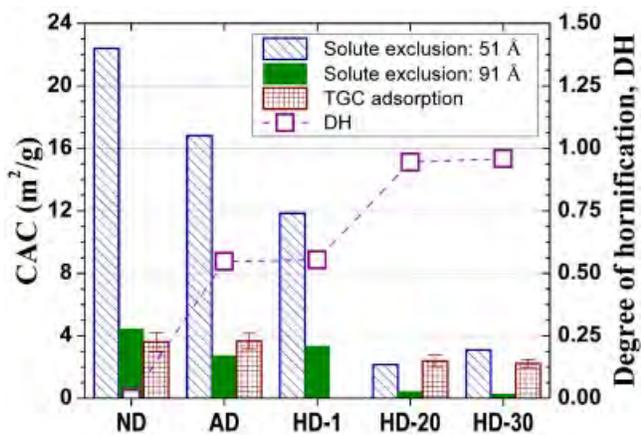
The solute exclusion technique can be used to reveal the phenomenon of pore collapse caused by fiber hornification by measuring pore volume distribution. The volumes of the pores with average diameter  $>30 \text{ \AA}$  of the dried substrates measured after rewetting were reduced compared with those of the ND substrate (Fig. 1). The reduction can be more than one order of magnitude when the substrate was heat dried for 20 min or more (the measurements were not able to resolve the difference between drying for 20 and 30 min as the pore volumes were so small). Air drying or heat drying



**Figure 1.** Pore volume distribution for a set of hornified substrates measured by the solute exclusion method.

for 1 min also caused the collapse of the pores as shown by the measured pore volume distributions (Fig. 1), especially those pores larger than 51 Å that are believed to be equal to the size of cellulase (Cowling and Kirk, 1976). The CACs of these hornified substrates along with the ND substrate can be determined from the pore volume distribution data using Equation (2). Two sets of CACs were determined assuming cellulase molecular size of 51 and 90 Å, respectively. The CACs determined using cellulase molecular size of 51 Å are certainly greater than those corresponding values determined using cellulase molecular size of 90 Å.

The results were compared with the CACs determined from TGC adsorption data (Fig. 2). Both methods show the



**Figure 2.** Cellulose accessibility to cellulase (CAC) of the same set of hornified substrates shown in Figure 1 measured by the solute exclusion and TGC adsorption methods, along with the degree of hornification (DH) of the substrates.

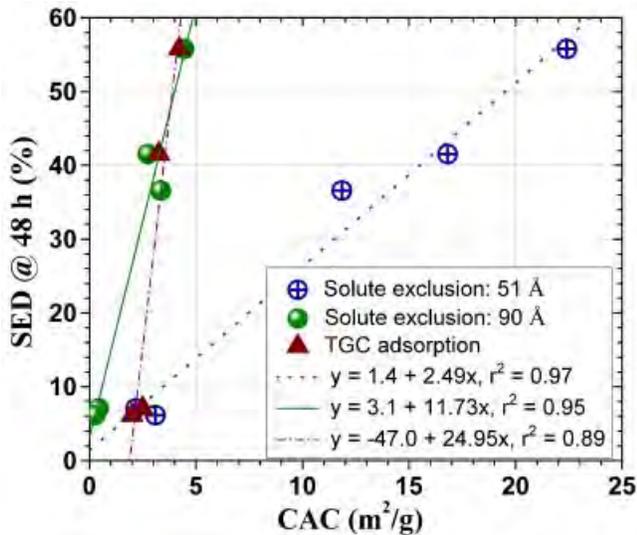
decrease of CAC by both air and heat drying. Furthermore, both methods show that CAC further decreased as drying duration was extended. However, the results in Figure 2 also show the discrepancies between the solute exclusion and TGC adsorption methods. The TGC adsorption method showed much smaller reduction in CAC after substrate experienced various degrees of drying than the solute exclusion method. The CACs for the HD-20 and HD-30 substrates were only reduced by 40% from its ND state, while the CACs of these two substrates determined from the solute exclusion method showed reductions by an order of magnitude. One explanation for the discrepancies is that the solute exclusion method does not account for substrate external surfaces while the TGC adsorption does. The measurement errors of both methods may also contribute to the differences observed. The TGC molecular weight is 62 kDa (Hong et al., 2007) or between 90 and 120 Å according to Table II. The CACs of the ND and AD substrates determined by the TGC adsorption were comparable to those using 90 Å. This further suggests that the TGC method is qualitatively in agreement with the solute exclusion method. The results in Figure 2 indicate that the probing molecular size does significantly affect the determination of CACs.

Fiber hornification was historically quantified by the percentage reduction in WRV (Jayme, 1944). We, therefore, also plotted the degree of fiber hornification (DH) of the substrates in Figure 2. DH was calculated using a modified definition to account for water not associated with fiber pores, but rather with fibrils as follows (Luo et al., 2011).

$$DH = \frac{WRV_{ND} - WRV}{WRV_{ND} - WRV_{CD}} \quad (3)$$

where  $WRV_{CD}$  is the measured WRV of the completed dried (hornified) substrate (oven dried for 24 h). DH of the ND substrate is zero and it is 1 for the completely dried (hornified) sample based on this definition. The results show that HD-20 and HD-30 substrates were almost completely hornified, suggesting that most of the pores were collapsed and inaccessible to cellulase as shown in Figure 1. This validates the lower values of CACs determined by the solute exclusion method than the TGC adsorption method that also account for substrate external cellulosic surfaces. The results in Figure 2 indicate that the reductions in CAC are directly correlated to the degree of hornification (DH).

To relate measured CAC to enzymatic hydrolysis, the substrate enzymatic digestibilities (SEDs) after 48 h hydrolysis were plotted against measured CACs (Fig. 3). The results indicate that SED is proportional to the measured CACs using both the solute exclusion and TGC adsorption method (Fig. 3). The linear relations suggest that CAC is the dominant factor affecting cellulose conversion. It should be emphasized that all substrates were from the same ND sample and therefore chemically identical. The fact that the reduction in SED from approximately 56% for the ND substrate (DH = 0) to approximately 37% for the HD-1

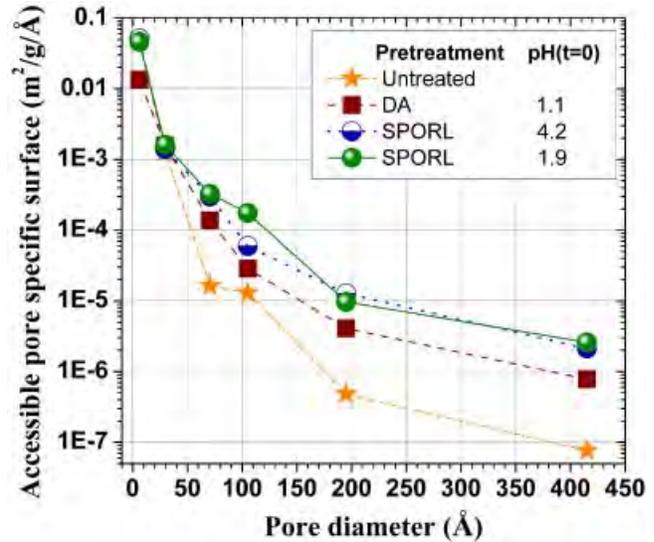


**Figure 3.** Correlations between substrate enzymatic digestibility (SED) and measured CACs by both the solute exclusion and TGC adsorption methods for the set of hornified substrates shown in Figure 1.

substrate (with only 1 min drying), or 34% reduction, indicates the significant impact of CAC on enzymatic hydrolysis efficiency. Because of nonlinear relationship between SED and DH, we used the SEDs of the two almost completely hornified substrates, HD-20 (DH = 0.945) and HD-30 (DH = 0.959) to linearly extrapolate the SED of 3.4% for a completely hornified substrate (DH = 1, all pores are not accessible to cellulase). Using the measured SED = 56% for the ND substrate (DH = 0), we can estimate that the pore surfaces contribute to 94% of the total hydrolysis, while the remaining 6% may be attributed to the substrate external surface, suggesting that the external surface plays a very small role in cellulose hydrolysis. This analysis is in agreement with our previous study that external surface only contributed to approximately 19% of the total hydrolysis of a bleached eucalyptus pulp (Luo and Zhu, 2011). Similarly, it is estimated that 88% of the accessibility of Avicel is contributed by its internal pore surfaces (Hong et al., 2007).

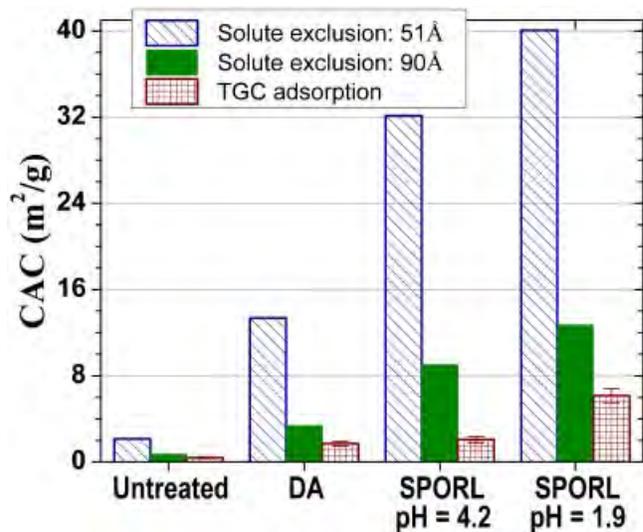
### Cellulose Accessibilities and Enzymatic Hydrolyses of Pretreated Substrates

Four substrates pretreated by different processes were used to further evaluate different techniques for CAC measurements. These substrates have different chemical compositions, as listed in Table I. Solute exclusion measurements indicate that different pretreatments produced different pore volume distributions (Fig. 4). The two SPORL pretreated substrates contain substantially more pore volume with pore size larger than 51 Å, accessible to cellulase, than the untreated and dilute acid (DA) pretreated



**Figure 4.** Pore volume distribution for a set of pretreated substrates by different pretreatment methods measured by the solute exclusion method.

substrates. The SPORL low pH (1.9) substrate contains even more volume in pores between 51 and 200 Å than the SPORL high pH (4.2). Similar to the results from the hornified substrates, the CAC determined from the solute exclusion method, especially that obtained using the 51 Å cellulase molecular size, is greater than that measured by the TGC adsorption method for a given substrate (Fig. 5). Recall the discussion using the hornified substrates, the molecular

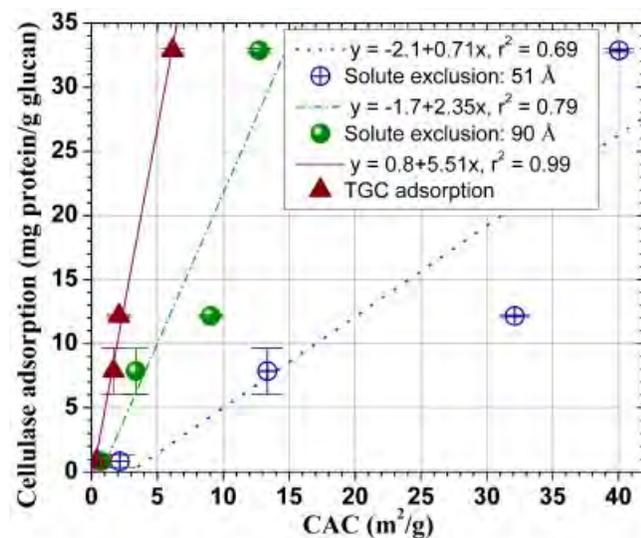


**Figure 5.** Comparisons of cellulose accessibility to cellulase (CAC) of the same set of pretreated substrates shown in Figure 4 measured by both the solute exclusion and TGC adsorption methods.

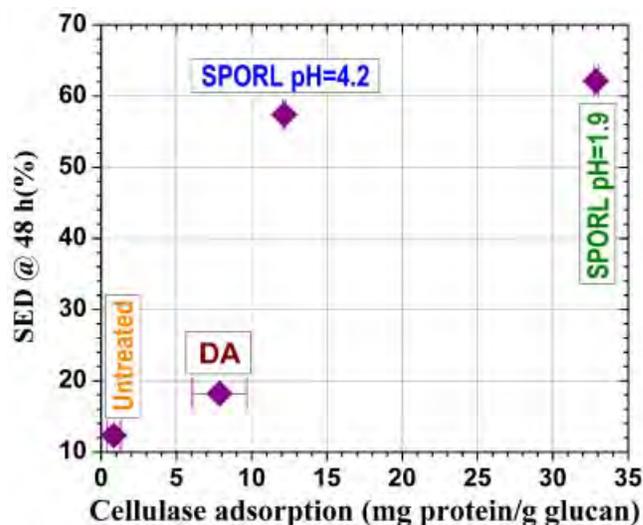
size of the TGC protein is most likely between 90 and 120 Å, this explains part of the differences in CACs among the three sets of data shown in Figure 5. The noncellulosic pore surfaces on the substrates also contribute to CACs determined by the solute exclusion method, and they are partially responsible for the differences between the solute exclusion and TGC adsorption measurements shown.

We also measured the amount of adsorptions of a commercial endoglucanase (exoglucanase deficient to be accurate), Fibercare<sup>®</sup>, onto these four substrates. As described in the experimental section, the measurements were conducted after adding 5 g/L of BSA in the substrate suspension to block nonproductive adsorption of cellulase by noncellulosic surfaces. The results indicate that the amounts of cellulase adsorption can correlate to the CACs measured by both the solute exclusion and TGC adsorption method (Fig. 6). In other words, the improved cellulose accessibility by pretreatment resulted in more significant increase in the cellulase adsorption (binding) to cellulose. The correlation with TGC adsorption measured CACs has a high correlation coefficient of 0.99, while the correlation coefficient with the 51 Å solute exclusion measurements was only 0.69, and with the 90 Å measurements was 0.79. This suggests that TGC adsorption mimics cellulase adsorption well. This may also suggest the inherent differences between the solute exclusion and protein adsorption method, in addition to measurement errors and the fact that solute exclusion includes noncellulosic surfaces.

The results of enzymatic saccharification of the four pretreated substrates were plotted against measured cellulase adsorption to illustrate the importance of cellulose



**Figure 6.** Correlations between the measured amounts of cellulase adsorption by the present UV-Vis spectrophotometry and CACs measured by both the solute exclusion and TGC adsorption methods.



**Figure 7.** The correlation of substrate enzymatic digestibilities (SEDs) and the amounts of cellulase adsorption measured by the present UV-Vis spectrophotometry for the set of pretreated substrates.

accessibility to enzymatic hydrolysis (Fig. 7). It was found that the increased cellulase adsorption due to improved CAC resulted in increased SED. The poor performance of the dilute acid (DA) pretreated substrate is due to the fact that pretreatment did not significantly increase CAC although the DA pretreatment removed almost all of the hemicelluloses and a similar amount of solids to those removed by the two SPORL pretreatments (Table I).

## Conclusions

Solute exclusion method using a set of probing molecules can provide substrate pore size distribution, which can be used to determine CAC provided that the cellulase molecular size is known. Direct pore volume probing method such as the solute exclusion method may over predict CAC by not accounting for noncellulosic surfaces. The TGC adsorption method is able to account for nonproductive adsorption of TGC using BSA to block noncellulosic surfaces. The molecular weight of TGC (62 kDa) needs to be taken into consideration when comparing CAC measured by TGC adsorption with other methods. Direct measurements of cellulase adsorption onto cellulosic surfaces using a commercial endoglucanase suggest that the amounts of cellulase adsorption are directly correlated to the CACs measured by the solute exclusion and TGC adsorption methods, demonstrating the validity of both methods for CAC evaluations.

CAC is the dominant factor to cellulose saccharification, as evidenced by the fact that when a substrate is completely hornified through drying, that is, all pores are collapsed and

not accessible to cellulase, the enzymatic hydrolysis efficiency can be reduced by approximately 94% compared to its ND state. Furthermore, the substrate enzymatic digestibilities are linearly proportional to the CACs of the hornified substrates. Based on the results from the hornified substrates, it was concluded that the substrate external surface has limited contribution to CAC, and it only accounts for approximately 6% of the cellulose hydrolysis efficiency. Pretreatment improved CAC by removing solids, thus increasing the substrate porosity. However, the effectiveness of pretreatment in terms of improving CAC varies significantly even when a similar amount of solids was removed. The increased CACs increased cellulase adsorption, and resulted in increased SED.

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